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CpG Oligonucleotides Can Prophylactically Immunize Against Th2-Mediated Schistosome Egg-Induced Pathology by an IL-12-Independent Mechanism¹

Mónica G. Chiaramonte,* Matthias Hesse,* Allen W. Cheever,[†] and Thomas A. Wynn^{2*}

Using a *Schistosoma mansoni* egg-induced granuloma model, we examined the ability of CpG oligodeoxynucleotides (ODN) to suppress Th2-type cytokine expression and to prophylactically immunize against Th2-dependent pulmonary pathology. The mechanism was examined by studying Th2 response regulation in cytokine-deficient mice. Surprisingly, our findings revealed several functions of CpG DNA that were completely IL-12 independent. Most striking was the marked suppression in Th2 cytokine expression and granulomatous inflammation observed in egg/CpG-sensitized IL-12-deficient mice. Immune deviation was not dependent on NK or B cells. However, a role for IL-10, B7.1, and CD40 expression in Th2 response inhibition was suggested. Indeed, CpG ODN up-regulated all three elements in both wild-type and IL-12-deficient mice. The role of IL-10 was demonstrated in mice exhibiting combined deficiencies in IL-12 and IL-10. Here, a marked increase in egg-specific IL-4/IL-5-producing cells confirmed a role for both cytokines in Th2 response inhibition. Nevertheless, the frequency of Th2-producing cells was again reduced by CpG ODN. However, in marked contrast to IL-12-deficient animals, a significant increase in IFN- γ -producing cells likely explains the reduced Th2 response in IL-10/IL-12-deficient mice. Thus, a novel IL-12-independent type 1-inducing pathway was revealed in the combined absence of IL-12 and IL-10. Together, these data demonstrate 1) that the Th1-promoting activity of CpG DNA is controlled by IL-12 and IL-10, and 2) that Th2 response inhibition by CpG ODN involves IL-12-independent changes in IL-10 and costimulatory molecule expression. These findings illustrate the utility of CpG DNA as adjuvants for vaccines designed to prevent Th2-dependent immunopathology. *The Journal of Immunology*, 2000, 164: 973–985.

The immunostimulatory activity of bacterial DNA was initially demonstrated in experiments showing the antitumor activity of *Mycobacterium bovis*-derived DNA (1). Unmethylated synthetic oligonucleotides with distinct base sequences (CpG motifs) triggered IFN- γ expression and activated NK cells (2), which in part explained the antitumor activity of the DNA. Since these initial observations, bacterial DNA and CpG motifs were shown to induce IL-12 and IFN- γ expression (3, 4) and to activate various immune cell subsets, including NK cells (5), macrophages (6, 7), dendritic cells (8, 9), as well as B cells (10). Recent findings suggest that CpG-induced IFN- γ secretion by NK cells is highly dependent on macrophage-derived IL-12 (6). Thus, immunostimulatory DNA sequences activate the immune system via a mechanism whereby macrophages and/or dendritic cells are initially targeted to produce IL-12. IL-12 in turn stimulates IFN- γ expression by NK and T cells, which promotes Th1 and suppresses Th2 cell development (11). CpG DNA-induced IFN- γ also affects B cells, enhancing Ig production (12), thus yielding a highly effective cell-mediated and humoral immune response.

Because CpG oligodeoxynucleotides (ODN)³ are strong inducers of IFN- γ and IL-12, they function as potent Th1 cell-promoting adjuvants. Indeed, they exhibited much of the same immunostimulatory activity (13, 14) previously described for rIL-12 (15). They function as effective immune adjuvants in tumor Ag immunization models (16) and enhance specific immunity for a growing list of important pathogens, including *Listeria monocytogenes* (17), influenza virus (18), hepatitis B virus (19) and *Leishmania major* (20). Protection in each case correlated with the induction of IL-12 expression and a Th1-type IFN- γ response (17).

Granuloma formation induced by the parasitic helminth *Schistosoma mansoni* is associated with a dominant Th2-type cytokine response (21, 22). Studies in gene knockout (KO) mice confirmed a central role for Th2 cytokines in mediating many aspects of the egg-induced inflammatory response (23–26), while rIL-12 almost completely suppressed both primary and secondary granuloma formation (27). Importantly, the reduction in granuloma size in IL-12-treated mice was associated with a shift in the Th2 cytokine response to one dominated by the production of IFN- γ (28). Experiments in IFN- γ -deficient mice demonstrated that the Th2-reducing ability of IL-12 was to a great extent attributable to IFN- γ from Th1 cells (29) and possibly NK cells (30).

As mentioned previously, IL-12 primarily targets NK cells and T cells and can stimulate Th1 cell differentiation in the absence of IFN- γ receptor signaling (31–33). CpG DNA targets multiple cell types and likely regulates Th1/Th2 cell development via mechanisms that are directly influenced by CpG-induced IL-12. Indeed, CpG DNA suppressed Th2-dependent pulmonary inflammation

*Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and [†]Biomedical Research Institute, Rockville, MD 20852

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² Address correspondence and reprint requests to Dr. Thomas A. Wynn, Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000 Rockville Pike, Building 4/126, Rockville, MD 20892-0425. E-mail address: twynn@niaid.nih.gov

³ Abbreviations used in this paper: ODN, oligodeoxynucleotide; KO, knockout; WT, wild type; SEA, soluble egg Ag; LALN, lung-associated lymph node; ELISPOT, enzyme-linked immunospot; HPRT, hypoxanthine phosphoribosyltransferase; PEC, peritoneal exudate cell.

(34, 35) in a manner similar to that described for IL-12 (27). Therefore, we examined the mechanism of this protective response by comparing the Th2-suppressing function of CpG DNA with that of rIL-12 in the schistosome egg pulmonary granuloma model. Specifically, IFN- γ , IL-12-, and IL-10-deficient mice were used to determine *in vivo* whether the Th2-inhibiting activity of CpG DNA was dependent on the induced expression of one or more of these cytokines. Because CpG DNA targets and activates B cells (10) and NK cells (2, 5), and both cell subsets can influence Th subset differentiation, we also examined their contribution to the Th2-reducing activities of CpG DNA. Surprisingly, the results from this study reveal an IL-12-, B cell-, and NK cell-independent Th2-suppressing mechanism, which appears to involve the CpG-induced expression of IL-10, B7.1, and CD40.

Materials and Methods

Animals

Six-week-old female mice, including wild-type (WT), IL-12 KO, IL-10 KO, IL-10/IL-12 KO, and IFN- γ KO mice, were C57BL/6 back-crossed and obtained from Taconic Farms (Germantown, New York). B cell-deficient (μ MT) and C57BL/10 control mice were also obtained from Taconic Farms. Animals were maintained in an American Association for the Accreditation of Laboratory Animal Care-approved animal facility.

Induction of pulmonary granulomas and sensitization procedures

The induction of synchronous egg-induced granulomas was performed as described (36). Briefly, *S. mansoni* eggs were isolated from the livers of infected mice (Biomedical Research Institute, Rockville, MD). To induce primary pulmonary granulomas, mice were injected with 5000 embryonated/mature eggs *i.v.* Secondary granulomas were induced in *i.p.* egg-sensitized mice (sensitized with 5000 eggs 2 wk previous to *i.v.* challenge). Animals were killed on days 3, 6, or 14 postchallenge. Some animals were sensitized in the presence of rIL-12 (a gift from Dr. Joe Sypek, Genetics Institute, Cambridge MA) administered by *i.p.* injection (0.25 μ g/day) on days 0, 1, 2, 3, and 5 (27). The sequence for the control ODN (non-CpG) was 5'-TGGATCCGACATGTCAGA-3' and was purchased from Oligos Etc (Wilsonville, OR). The sequence for the CpG ODN 1826 was 5'-TCCATGACGTTCTGACGTT-3' and was kindly provided by Dr. Arthur M. Krieg (University of Iowa, Iowa City, IA). ODN were administered by *i.p.* injection (5 μ g/day/mouse) using a protocol identical with the rIL-12 regimen. In the NK cell depletion experiments mice were treated with 1.2 mg anti-asialo GM ϕ *i.p.* on days -4 and -1 during sensitization. This depleted >97% of NK cells as determined by FACS analysis of spleen cells.

Histopathology and serology

For measurement of granulomas, the left lung was inflated with Bouin-Hollande fixative and processed routinely. The size and cell composition of the pulmonary granulomas was determined in histological sections stained by Wright's Giemsa stain. The diameters of ~30 granulomas containing a single egg were measured with an ocular micrometer, and the volume of each granuloma was calculated assuming a spherical shape. The percentage of eosinophils was evaluated in the same sections. The same individual who had no knowledge of the experimental design scored all histological examinations.

Total IgE in serum was determined using a sandwich ELISA (PharMingen, San Diego, CA) and quantified according to a reference IgE standard (anti-trinitrophenyl IgE, PharMingen). Plates were coated with anti-mouse IgE capture mAb from clone R35-72 in 0.1 M NaHCO $_3$, pH 8.2, overnight at 4°C. The secondary mAb was biotinylated anti-mouse IgE from clone R35-92. The streptavidin-peroxidase reagent was diluted 1:1000 in PBS/1% BSA. Isotype-specific Ab titers were evaluated by indirect ELISA. Immulon 4 plates were coated with 10 μ g/ml of soluble egg Ag (SEA) (50 μ l/well) diluted in PBS, and serum samples were analyzed using serial 2-fold dilutions. Second-step HRP-conjugated rabbit anti-mouse IgG, IgG1, IgG2a, and IgG2b Abs (Zymed Laboratories, San Francisco, CA) were employed at a 1:1000 dilution. The absorbance was read at 405 nm using a Vmax Kinetic Microplate Reader (Molecular Devices, Palo Alto, CA) after adding 100 μ l of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid):H $_2$ O $_2$ one-step substrate.

Cytokine assays

In vitro cytokine measurements, single-cell suspensions of lung-associated lymph nodes (LALN) were prepared aseptically at various times after injection of schistosome eggs. LALN were pooled from four to five animals. Cells were plated in 24-well tissue culture plates at a final concentration of 3×10^6 cells per ml in RPMI 1640 supplemented with 2 mM glutamine, 25 mM HEPES, 10% FCS, 50 μ M 2-ME, penicillin, and streptomycin. Cultures were incubated at 37°C in an atmosphere of 5% CO $_2$ in air. Cells were stimulated with SEA at 20 μ g/ml or with 5 μ g/ml Con A. In some experiments, anti-CD4 was also added (50 μ g/ml). Supernatants were harvested at 72 h. IFN- γ and IL-5 were measured by two-site ELISA as previously described (28). IL-4 levels were determined by proliferation of CT4S cells. Cytokine levels were calculated using standard curves constructed using recombinant-murine cytokines.

Enzyme-linked immunospot (Elispot) assay

Single-cell suspensions of LALN were plated in 24-well plates (3×10^6 cells/ml) and stimulated with SEA (20 μ g/ml). After 24 h, cells were harvested and plated (1×10^5 , 5×10^4 , and 1×10^4 cells per well) in 96-well plates previously coated with different cytokine-specific Abs (anti-IFN- γ , anti-IL-4, anti-IL-5) and subsequently blocked with 5% FCS (37). Cells were incubated overnight at 37°C in an atmosphere of 5% CO $_2$. After washing with PBS/Tween 20, plates were incubated with biotinylated anti-cytokine Ab (1/1000 dilution) for 2 h at 37°C. Plates were washed and incubated with alkaline phosphatase avidin (1/5000 dilution) 1 h at room temperature. Plates were developed using 5-bromo-4-chloro-3-indolyl phosphate-agarose, and spots were counted the following day using an inverted microscope.

RT-PCR detection of cytokine mRNAs

A RT-PCR was performed to determine relative quantities of mRNA for IFN- γ , IL-10, and hypoxanthine phosphoribosyltransferase (HPRT). Reverse transcription of 1 μ g of RNA was performed as described (36). The primers and probes for all genes have been published (27, 36, 38).

After the appropriate number of PCR cycles, the amplified DNA was analyzed by electrophoresis, Southern blotting, and hybridization with non-radioactive cytokine-specific probes as previously described (36). The chemiluminescent signals were quantified using a 600 ZS scanner (Molecular Dynamics, Torrance, CA). Results were normalized for the relative quantity of total mRNA by comparison to HPRT (39).

In vitro production of IL-10/IL-12 by CpG-activated peritoneal exudate cells (PECs)

Mice were injected with 2 ml sterile thioglycollate, and, 4 days later, PECs were collected by lavage. Cells were plated (5×10^6 cells/well) in 24-well plates and allowed to adhere for 2 h. Nonadherent cells were removed by vigorous washing. The adherent cells were treated with 2 μ g/ml CpG DNA for 0, 6, 12, and 24 h, and culture supernatants were assayed by ELISA for IL-10 and IL-12 p40.

Flow cytometry

Mice were injected with 30 μ g of CpG ODN *i.p.* (total dose used in all sensitization experiments) 4 days after being injected with thioglycollate *i.p.* PECs were harvested 24 h after CpG injection. Cells (5×10^5) were washed in PBS containing 0.5% BSA/0.02% NaN $_3$ at 4°C and incubated 10 min at 4°C with anti-Fc γ RIII Ab (PharMingen) to block nonspecific binding of the following reagents. FITC-anti I-A b (AF6-120.1), FITC-anti-mouse B7.1/CD80, FITC-anti-mouse B7.2/CD86, PE-anti-CD40, and biotinylated rat anti mouse-F4/80 Ag (clone CI:A3-1) was used at 5 μ g/ml. FITC and PE isotype controls were included. Between all incubation steps (30 min, 4°C), cells were washed with PBS/FCS. FACS analysis was performed on a FACScalibur cytofluorometer (Becton Dickinson, San Diego, CA), acquiring 10,000 events. FACS data were analyzed using CellQuest (version 3.1; Becton Dickinson).

Statistics

Values of $p \leq 0.05$ determined by ANOVA or by Student's *t* test were considered significant. All experiments shown were repeated two to five times with similar results.

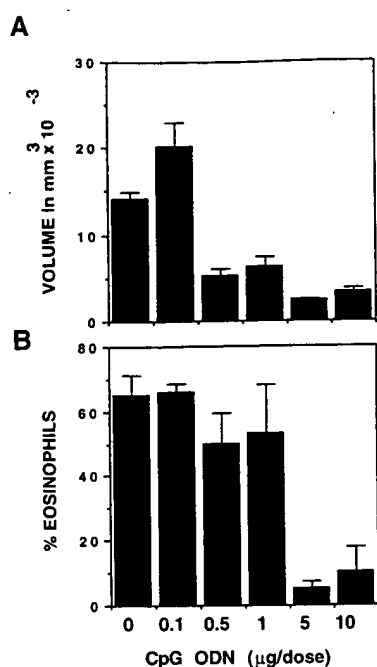


FIGURE 1. Increasing doses of CpG ODN administered as an adjuvant reduces subsequent schistosome egg-induced lung pathology. CpG ODN was injected i.p. on days 0, 1, 2, 3, and 5 following i.p. egg sensitization. Two weeks later, animals were challenged i.v. with 5000 eggs and sacrificed on day 8. *A*, The size of granulomas was determined in Giemsa-stained lung sections and reported as the mean value \pm SEM of four to five mice per group. *B*, The cellular composition of individual granulomas was evaluated and the mean percentage of eosinophils \pm SEM of four to five mice per group is reported.

Results

CpG oligonucleotides administered with parasite Ag prophylactically immunize against schistosome egg-induced pulmonary granuloma formation

CpG ODN serve as vaccine adjuvants that enhance Th1-type CD4⁺ T cell immunity (40). In these experiments, we examined the mechanisms behind the adjuvant activity of CpG DNA and focused on their ability to immunize against a Th2-dependent inflammatory response of the lung. Multiple doses of CpG ODN were administered using a similar protocol to that described for rIL-12 (27). Mice were sensitized i.p. with purified schistosome eggs \pm CpG ODN as described in *Materials and Methods*. Two weeks later, animals were challenged i.v. with 5000 freshly isolated eggs, and granuloma formation was evaluated after 8 days. Granuloma size was significantly decreased with a sensitizing protocol employing as little as 0.5 µg/dose of CpG ODN (Fig. 1A). Granuloma size was maximally decreased in mice receiving the 5-µg dose. Only the 5- and 10-µg doses significantly decreased the tissue eosinophilia (Fig. 1B). Administration of a control ODN (non-CpG-containing motif) had no inhibitory effect on granuloma size, cellular composition, or Th2 cytokine response when compared with the saline-treated mice (data not shown). In one experiment where control ODN was directly compared with CpG ODN, granuloma size was 20.4 ± 1.6 and 4.9 ± 0.8 in the control and CpG groups, respectively.

LALN cells were isolated and restimulated in vitro with SEA or Con A. IFN- γ in culture supernatants was used as a marker of a Th1-type response and IL-5 and IL-10 as markers of a Th2 response (Fig. 2A). As expected, the egg-sensitized/saline-treated

control group showed a highly polarized Th2-type response with high levels of IL-5 and IL-10 but little or no Ag-specific IFN- γ . There was a dose-dependent decrease in the expression of the Th2-associated cytokines and an increase in IFN- γ production with increasing amounts of CpG ODN. Indeed, in complete agreement with granuloma and eosinophil results, the 5-µg dose resulted in a maximal suppression of the Th2-type cytokine response. Splenocyte cultures showed a similar profile (data not shown). Because granuloma size, tissue eosinophilia, and Th2-type cytokines were maximally reduced employing the 5-µg dose, this concentration was used in all subsequent experiments.

CpG oligonucleotides modulate the Ab isotype profile in egg-challenged mice but suppress Th2-mediated pathology in the complete absence of B cells

Sera were collected from mice at the time of sacrifice and assayed for anti-SEA Ig. IgG1 and IgG2b Ab isotypes were examined as markers of a Th2- or Th1-polarized response, respectively (Fig. 2B). Total serum IgE was also measured in egg/saline- and egg/CpG ODN-sensitized mice and compared with levels in animals sensitized with eggs and rIL-12. Consistent with previous results demonstrating that schistosome eggs preferentially induce a Th2 response (41), IgG1 (Fig. 2B) and IgE (data not shown) Abs dominated the response in egg/saline-treated mice. In agreement with the increased IFN- γ response, CpG ODN down-regulated SEA-specific IgG1 and up-regulated IgG2b titers. Moreover, total IgE levels were decreased to levels comparable with the egg/rIL-12-treated group (not shown).

Although B cells, in contrast to professional APCs like dendritic cells, are to a large extent dispensable during the priming of immune responses (42), CpG ODN were shown to directly induce murine B cells to proliferate and secrete Ig in vitro and in vivo (10). Therefore, B cells may participate in the establishment of CpG-induced Th1 responses. To determine whether B cells were required for the generation of the egg-specific Th1 response, we sensitized WT and B cell-deficient mice (μ MT) with eggs in the presence or absence of CpG ODN or IL-12. As shown in Fig. 3, granuloma size and the associated tissue eosinophilia were decreased similarly by CpG DNA or IL-12 administration in both WT and B cell-deficient mice, indicating that there was no specific B cell requirement for CpG ODN-mediated effects.

The prophylactic inhibition of egg-induced pathology by CpG oligonucleotides is to a large extent IL-12 independent, but is highly IFN- γ dependent

Previous studies demonstrated a strict requirement for IFN- γ in the prophylactic inhibition of granuloma formation and Th2 responses by rIL-12 (41). Because CpG DNA targets professional APCs such as dendritic cells and macrophages (8, 9), and triggers IL-12 production, we determined whether the CpG-induced suppression of the Th2 response was dependent on endogenous IL-12 and/or IFN- γ . The activity of rIL-12 was compared with CpG ODN in animals deficient in either IFN- γ or IL-12 p40. WT and cytokine-deficient mice were sensitized with eggs alone (saline) or eggs with rIL-12 or CpG ODN and then challenged with eggs i.v. 2 wk later. As expected, both rIL-12- and CpG ODN-treated WT mice displayed a highly significant suppression in granuloma formation (Fig. 4A) and tissue eosinophilia (Fig. 4B) as a result of the sensitization protocols. Consistent with previously published results (41), rIL-12 failed to affect granuloma formation in the absence of endogenous IFN- γ and CpG ODN displayed a similar impairment. Surprisingly, granuloma size was consistently and significantly down-regulated in the absence of endogenous IL-12, demonstrating that there is no specific requirement for IL-12 in the inhibition

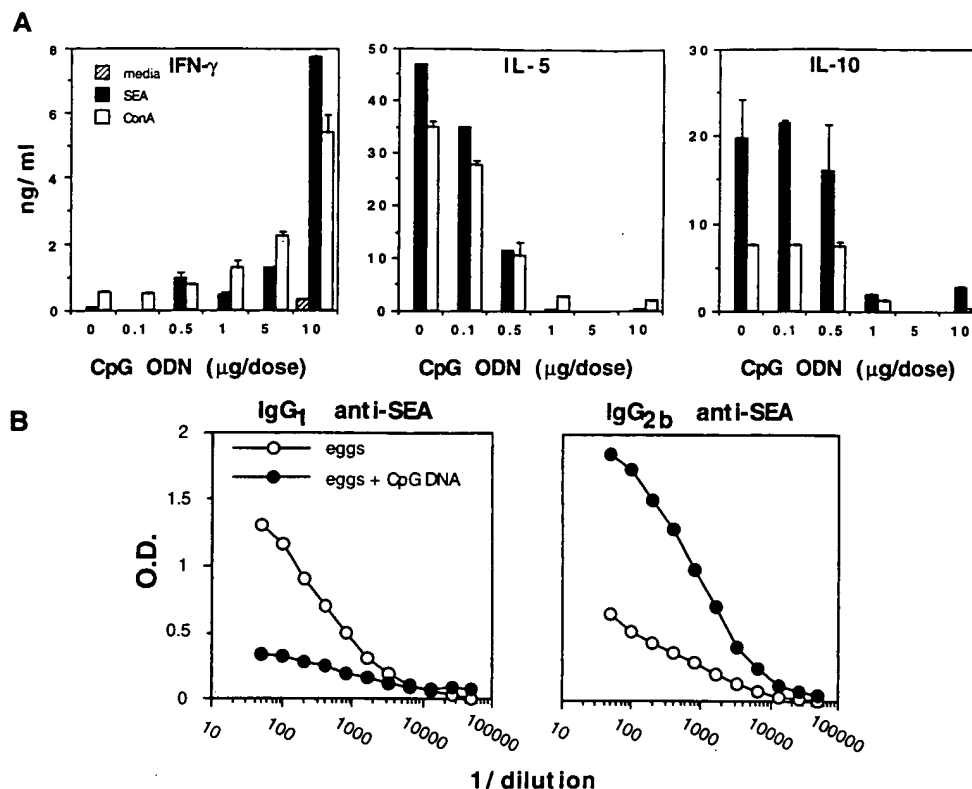


FIGURE 2. The CpG ODN-induced reduction in granuloma formation correlates with an increase in Th1 and decrease in Th2-associated cytokine/Ab expression. Mice were sensitized as described in Fig. 1. A, LALN cell suspensions prepared on day 8 were placed in culture (3×10^6 cells/well) and restimulated with SEA (20 μ g/ml) or Con A (5 μ g/ml). Supernatants were collected at 72 h, and IFN- γ , IL-5, and IL-10 were measured by ELISA. Graphics illustrate the means of supernatants analyzed in duplicate. B, Mice (five per group) were bled on day 8 and SEA-specific Ab titers measured by ELISA. Each curve represents the mean values of pooled sera from each group.

of granulomatous inflammation by CpG ODN. Clearly, however, the effect was more complete in the presence of IL-12. Interestingly, while granuloma volumes were markedly decreased in IL-12-deficient mice by CpG ODN, the overall cellular phenotype of the lesions was not significantly different as there was little or no change in the tissue eosinophilia by CpG ODN (Fig. 4B). This is in contrast to WT mice, where both size and tissue eosinophilia was affected.

CpG oligonucleotides fail to up-regulate an Ag-specific IFN- γ response in the absence of endogenous IL-12 but nevertheless decrease Th2-type cytokine expression

The Th1/Th2 cytokine balance significantly influences the cellular composition of schistosome egg-induced granulomas, with IL-5 promoting tissue eosinophilia and IFN- γ a significant inhibitory factor (41, 43). Because the granulomas of CpG ODN-sensitized IL-12-deficient mice were decreased in size, yet displayed a similar cellular phenotype as the saline-treated controls, we wanted to determine whether the deviated immune response observed in WT animals (Fig. 2A) was also occurring in the CpG DNA-treated IL-12-deficient mice. For these studies, WT, IFN- γ KO, and IL-12 KO mice were sensitized with eggs and either CpG ODN or IL-12 as described above. Eight days following i.v. egg challenge, animals were sacrificed and LALN cells isolated, placed in culture, and stimulated with SEA or mitogen. As expected, WT mice displayed a polarized Th2-type response, showing high levels of IL-5 and no IFN- γ in response to SEA (Fig. 5). CpG ODN and IL-12 increased IFN- γ expression and decreased IL-5 levels in the WT mice. Also in agreement with previous observations, rIL-12 failed

to down-regulate Ag-specific IL-5 production in the absence of endogenous IFN- γ (33, 41). CpG ODN was also ineffective although there was a slight decrease in the experiment shown (Fig. 5). Interestingly, however, CpG ODN, despite failing to induce an Ag-specific IFN- γ response, markedly reduced IL-5 production in the absence of endogenous IL-12. Recombinant IL-12, in contrast, up-regulated IFN- γ expression and simultaneously reduced IL-5 levels in the IL-12 KO animals. These data suggested that CpG ODN might decrease Th2 responses in IL-12 KO mice via a mechanism distinct from IFN- γ -mediated cross-regulation. Such a mechanism might explain the failure to inhibit the tissue eosinophilia in the CpG ODN-treated IL-12-deficient mice (Fig. 4B). The maintenance of a reduced but significant IL-5 response in the absence of a CpG-induced type 1 response would certainly contribute to such an outcome (Fig. 5). Thus, the overall magnitude of the Th2-type response is reduced by CpG ODN but not "deviated" to type 1 in the absence of endogenous IL-12.

The prophylactic inhibition of granuloma formation in IL-12-deficient mice by CpG oligonucleotides is not dependent on the presence of asialo-GM $_1$ ⁺ NK Cells

CpG ODN activate NK cells and enhance their cytolytic activity (5). Thus, although CpG ODN failed to promote an Ag-specific IFN- γ response in the absence of IL-12 (Fig. 5), a transient up-regulation of IFN- γ production by CpG ODN-activated NK cells (6) during the period of egg/CpG ODN sensitization might provide an explanation for the reduced Th2-type responses in these animals. To address this hypothesis, we depleted NK cells during i.p. egg/CpG ODN sensitization in WT and IL-12-deficient mice. As

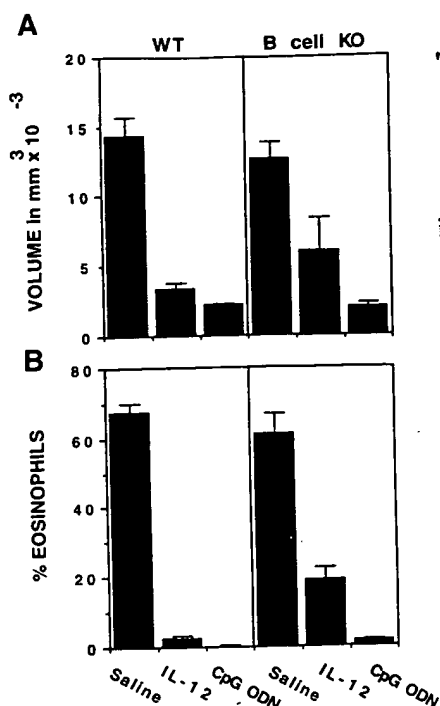


FIGURE 3. Suppression of egg-induced pathology by egg/CpG ODN sensitization occurs in the absence of B cells. WT C57BL/10 and B cell-deficient mice (μ MT) were sensitized i.p. with eggs alone, eggs + rIL-12 (0.25 μ g), or eggs + CpG ODN (5 μ g) 2 wk before i.v. egg injection and sacrificed on day 8. The left lung from each animal was examined histologically to evaluate the size of granuloma (A) and tissue eosinophilia (B). Each bar represents the mean \pm SEM of five mice/group.

expected, CpG ODN markedly decreased granuloma size in both WT and IL-12-deficient mice when compared with the saline-treated controls (Fig. 6A). Also, consistent with previous results, the tissue eosinophilia was not reduced in the absence of IL-12 (Fig. 6B) but was nearly ablated in the CpG ODN-treated WT mice. Interestingly, however, NK cell depletion had no significant effect on the granulomatous response or tissue eosinophilia in either CpG-treated WT or IL-12-deficient mice, suggesting that these cells play little or no role in the inhibition of the Th2-dependent inflammatory response. Similar findings were also observed when rIL-12 was substituted for CpG ODN (data not shown).

Granuloma size is suppressed by CpG ODN during a primary schistosome egg-challenge and associated with a marked up-regulation of IFN- γ and IL-10 mRNA in the lung

Studies with recombinant IL-12-treated mice showed that in addition to promoting a Th1-type IFN- γ response, IL-10 is also up-regulated in vivo (27, 44). With some intracellular pathogens, this IL-10 response is critical for regulating the protective but potentially host-damaging Th1-type response (45, 46). We demonstrated that endogenous IL-10 plays a similar inhibitory role during Th2-type responses (25, 37). Thus, the down-regulation of granuloma formation by CpG ODN in the absence of IL-12 might be at least in part mediated through IL-10. To examine this question, we first examined whether CpG ODN induced IL-10 production in a manner similar to that previously reported for rIL-12 (44). For these studies, naive mice were challenged i.v. with schistosome eggs and treated with either rIL-12 or CpG ODN, and IFN- γ and IL-10 mRNA in the lung was examined at several time points. As shown in Fig. 7A, the peak IFN- γ mRNA response occurs on day 3 post-

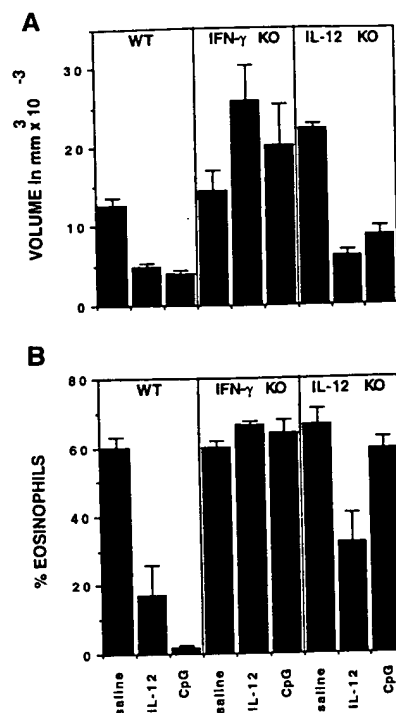


FIGURE 4. Suppression of egg-induced pathology by egg/CpG ODN sensitization is not dependent on endogenous IL-12. WT, IFN- γ KO, and IL-12 p40 KO mice were sensitized i.p. with eggs alone, eggs + rIL-12 (0.25 μ g), or eggs + CpG ODN (5 μ g) 2 wk before i.v. egg injection and sacrificed on day 8. Granuloma size (A) and tissue eosinophilia (B). Each bar represents the mean \pm SEM of five mice/group.

challenge and then declines to baseline levels by day 14 in the saline-treated egg-injected controls. An increase in IL-10 mRNA expression follows the rise in IFN- γ , peaks on day 6, and is reduced at later time points. By day 14, as has been described extensively in related reports (36, 47), the immune response is characterized by the dominant expression of several Th2-associated cytokines (data not shown). There was a marked up-regulation of IFN- γ and IL-10 mRNA in the rIL-12-treated animals. Interestingly, the CpG ODN-treated mice displayed a similar induction of IFN- γ and IL-10 mRNA, although the peak response was somewhat delayed when compared with the rIL-12-treated mice. In agreement with the secondary challenge studies described above, primary granuloma size was also decreased in both CpG-treated WT and IL-12-deficient mice (Fig. 7B), but tissue eosinophilia was significantly reduced only in the WT mice.

Selective up-regulation of IL-10 or IL-12 p40 expression in CpG-activated macrophages from IL-12-deficient or IL-10-deficient mice, respectively

In subsequent experiments, we examined if CpG ODN directly stimulated IL-10 and IL-12 p40 expression in macrophages and whether IL-10 was selectively up-regulated in the absence of IL-12. For these experiments, thioglycollate-elicited peritoneal macrophages from WT, IL-10 KO, IL-12 KO, and IFN- γ KO mice were stimulated in vitro with CpG ODN for 6, 12, and 24 h, and the culture supernatants were examined for IL-10 and IL-12 p40 levels by ELISA. Similar to the results in vivo (Fig. 7A), WT mice showed a simultaneous up-regulation of IL-10 and IL-12 in response to CpG ODN (Fig. 8). Interestingly, while CpG-induced IL-12 p40 levels were dramatically elevated in the IL-10 KO vs

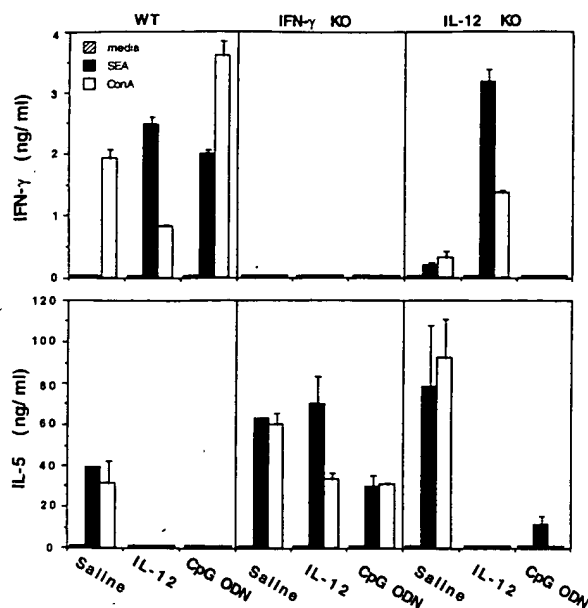


FIGURE 5. CpG ODN fail to up-regulate SEA-specific IFN- γ response in the absence of IL-12 but nevertheless dramatically decrease Th2-type cytokine expression. WT, IFN- γ KO, and IL-12 p40 KO mice were treated as described in Fig. 4. Single-cell suspensions were prepared from isolated LALN, placed in culture, and restimulated with SEA (20 μ g/ml) or Con A (5 μ g/ml). IFN- γ and IL-5 levels were measured by ELISA in the 72-h culture supernatants. Bars represent means of supernatants analyzed in duplicate.

WT cultures, there was no additional increase in IL-10 levels in the absence of endogenous IL-12, suggesting that while IL-10 regulates IL-12 p40 production, there is no evidence for cross-regulation of IL-10 by IL-12. This is likely explained by the differential responsiveness of macrophages to IL-10 vs IL-12. Similar to the WT mice, there was a simultaneous induction of IL-10 and IL-12 p40 in IFN- γ -deficient mice, although the levels of IL-10 were consistently and significantly lower in the IFN- γ KO vs WT or IL-12 KO macrophage cultures.

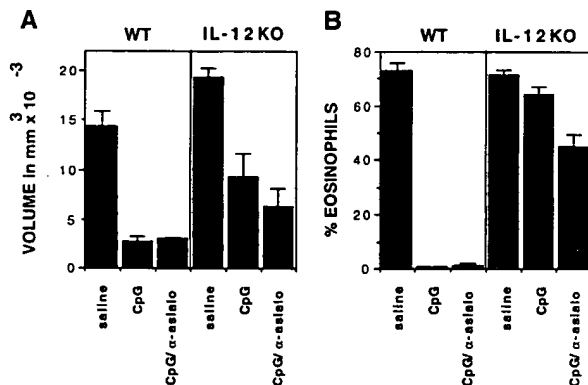


FIGURE 6. Suppression of egg-induced pathology by egg/CpG ODN sensitization in IL-12-deficient mice is not dependent on the presence of asialo-GM $_1$ NK cells. WT and IL-12 p40 KO mice were sensitized with eggs or eggs plus CpG ODN (5 μ g). NK cells were depleted during sensitization with anti-asialo GM $_1$ Abs. Mice were i.v.-challenged 2 wk later. Five mice per group were sacrificed on day 8, and the mean granuloma volume and percentage of eosinophils determined in lung sections.

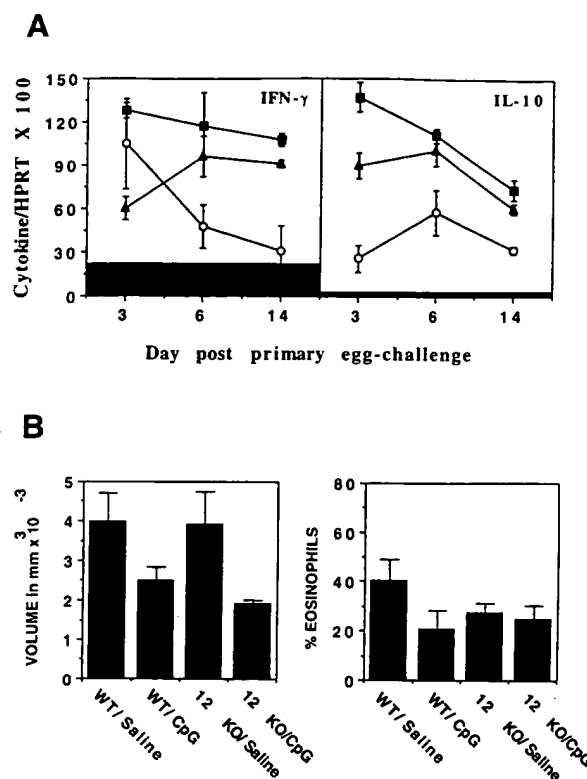


FIGURE 7. CpG ODN simultaneously induces IFN- γ and IL-10 mRNA expression in the granulomatous tissues. A, Naive WT mice were injected i.v. with schistosome eggs and treated with saline (\square), CpG ODN (\blacktriangle), or rIL-12 (\blacksquare) on days 0, 1, 2, 3, and 5 after i.v. challenge. Mice were sacrificed on different days postchallenge as indicated. Each lung was placed in RNA-STAT60 for mRNA extraction, and cytokine mRNA levels were measured by RT-PCR and expressed as arbitrary OD units of cytokine mRNA/HPRT mRNA (housekeeping gene). The values shown are the mean OD values \pm SEM of three to five mice per group. The shaded bars depict the cytokine mRNA levels observed in naive unchallenged mice. B, WT and IL-12 p40 KO mice were treated as described in A and sacrificed on day 6. Granuloma size and eosinophil numbers were determined in Giemsa-stained lung sections. Bars represent mean values \pm SEM of four to five mice per group.

CpG ODN decreases Th2-dependent pathology in the absence of IL-10 or IL-12, but to a lesser extent in mice simultaneously deficient in both IL-12 and IL-10

Because IL-10 (25) and IL-12-induced IFN- γ (41) both inhibited Th2 responses, we used mice with deletions in IL-10 and IL-12 to determine whether blocking both Th2 inhibitory pathways would affect the anti-inflammatory activity of CpG ODN, which is maintained in the absence of endogenous IL-12 (Fig. 4). Mice with individual or combined deficiencies were sensitized with eggs in the presence or absence of CpG ODN. As shown in Fig. 9, egg/CpG-sensitized WT, IL-10-, and IL-12-deficient mice all showed significant reductions in granuloma size. Although the CpG-sensitized double cytokine-deficient mice also exhibited a decrease in granuloma size, the result was insignificant when compared with the saline-treated control groups.

CpG oligonucleotides induce IFN- γ expression by an IL-12-independent pathway in mice that are deficient in both IL-12 and IL-10

To examine the cytokine response in detail, we performed ELISPOT assays on LALN cells to ascertain whether CpG ODN modulated

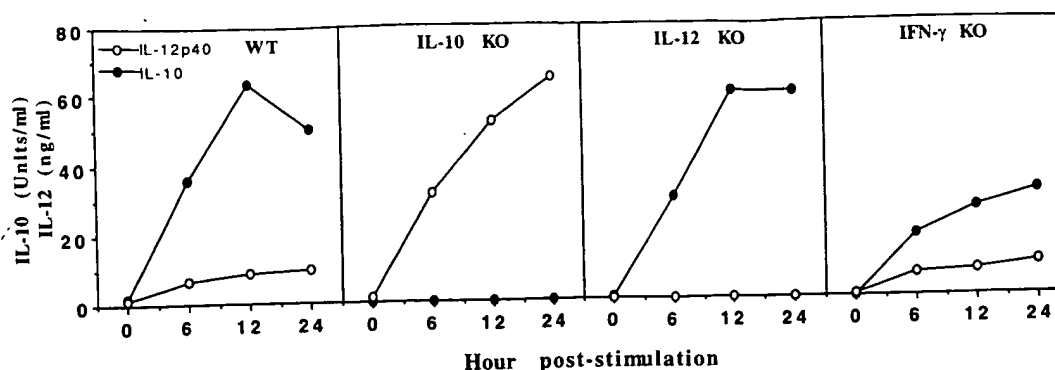


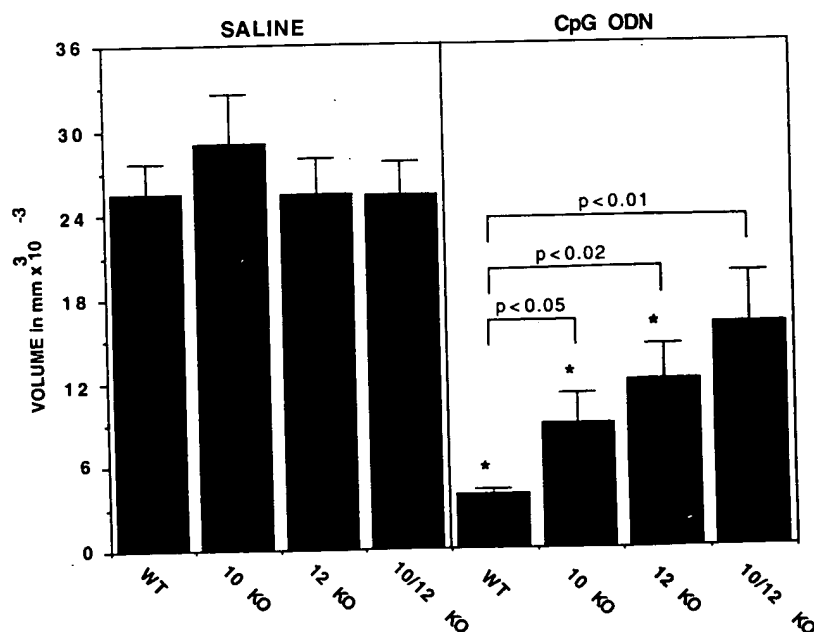
FIGURE 8. There is a selective up-regulation of IL-10 or IL-12 p40 expression in CpG ODN-activated macrophages derived from IL-12 KO or IL-10 KO mice, respectively. Thioglycollate-elicited macrophages from WT, IL-12 KO, IL-10 KO, and IFN- γ KO mice were cultured *in vitro* (5×10^6 well) and stimulated with CpG ODN ($2.0 \mu\text{g}$) for 0, 6, 12, or 24 h. Supernatants were collected and assayed by ELISA for IL-10 and IL-12 p40.

the frequencies of SEA-specific IFN- γ , IL-4-, and IL-5-producing cells in the various cytokine-deficient animals. As might be expected, CpG-treated WT mice showed a marked increase in IFN- γ -producing cells and a highly significant reduction in the number of IL-4 and IL-5 producers (Fig. 10). IL-10-deficient mice displayed a similar profile, although there was a more dramatic increase in the number of IFN- γ -producing cells and, in contrast to the WT animals, CpG ODN failed to completely ablate IL-4 producers. Consistent with the cytokine ELISA results reported above (Fig. 5), IL-12-deficient mice showed no increase in IFN- γ -producing cells as a result of CpG ODN treatment, although the frequencies of IL-4- and IL-5-producing cells were decreased. However, the most surprising findings from this experiment were the results obtained with the double IL-10/IL-12-deficient mice. These mice showed the most dramatic increase in IL-4- and IL-5-producing cells when compared with the saline-treated WT, IL-10-, or IL-12-deficient mice, but nevertheless again displayed reduced numbers when sensitized in the presence of CpG ODN. Perhaps even more intriguing, the CpG-treated IL-10/IL-12-deficient mice, in contrast to the single IL-12-deficient mice, displayed an IL-12-independent increase in IFN- γ -producing cells. Both of these

changes could explain the slight decrease in granuloma size observed in the egg/CpG-sensitized double KO mice.

The culture supernatants were also examined by ELISA, and, consistent with the ELISPOT data, lymphocytes obtained from CpG ODN-treated WT and IL-10 KO produced large quantities of IFN- γ in response to SEA stimulation (Fig. 11A). Indeed, even the egg/saline-sensitized IL-10 KO mice produced a significant amount of IFN- γ . In contrast, lymphocytes from IL-12-deficient mice showed no SEA-specific IFN- γ response. Again, consistent with the ELISPOT data, the double IL-10/IL-12-deficient mice displayed an up-regulation in IFN- γ expression that was similar to WT animals. Addition of anti-CD4 mAbs to the Ag-stimulated cultures significantly suppressed the production of IFN- γ , indicating that the response was largely CD4 $^+$ T cell dependent (data not shown). We also examined the IFN- γ mRNA response *in vivo*. In agreement with the ELISPOT and ELISA data, only the WT, IL-10-, and double IL-10/IL-12-deficient mice displayed increased IFN- γ mRNA levels in the lung when sensitized in the presence of CpG ODN (Fig. 11B). The latter findings provide further evidence of an IL-12-independent IFN- γ -inducing pathway in the double IL-10/IL-12-deficient mice.

FIGURE 9. Suppression of egg-induced pathology by egg/CpG ODN sensitization in IL-12 KO, IL-10 KO, and double IL-10/IL-12-deficient mice. WT, IL-10, IL-12, and IL-10/IL-12-deficient mice were sensitized as described in Fig. 1. All animals were *i.v.* challenged 2 wk later. Mice were sacrificed on day 8. Data were pooled from two experiments and include a total of 8–10 mice per group. *, Significantly different from the saline-treated control groups ($p \leq 0.05$ by Student's *t* test). Additional statistically significant comparisons and their *p* values are included within the figure.



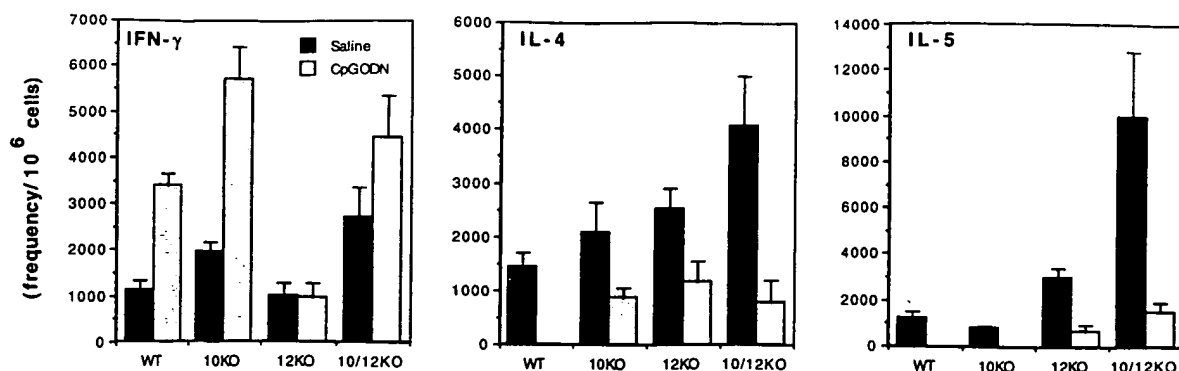


FIGURE 10. ELISPOT assays reveal the frequency of Ag-specific IFN- γ , IL-4, and IL-5-producing cells in egg/CpG-sensitized WT, IL-10, IL-12, and IL-10/IL-12-deficient mice. WT, IL-10-, IL-12-, and IL-10/IL-12-deficient mice were sensitized as described in Fig. 9. LALN were isolated after i.v. egg challenge, placed in culture, and restimulated with SEA (20 μ g/ml). Cells were harvested and placed in 96-well plates coated with anti-cytokine Ab. ELISPOT assay were performed as described in *Materials and Methods*. Bars represent the mean number of cytokine producing per 10^6 cells from each group \pm SD (six mice per group), analyzed in duplicate.

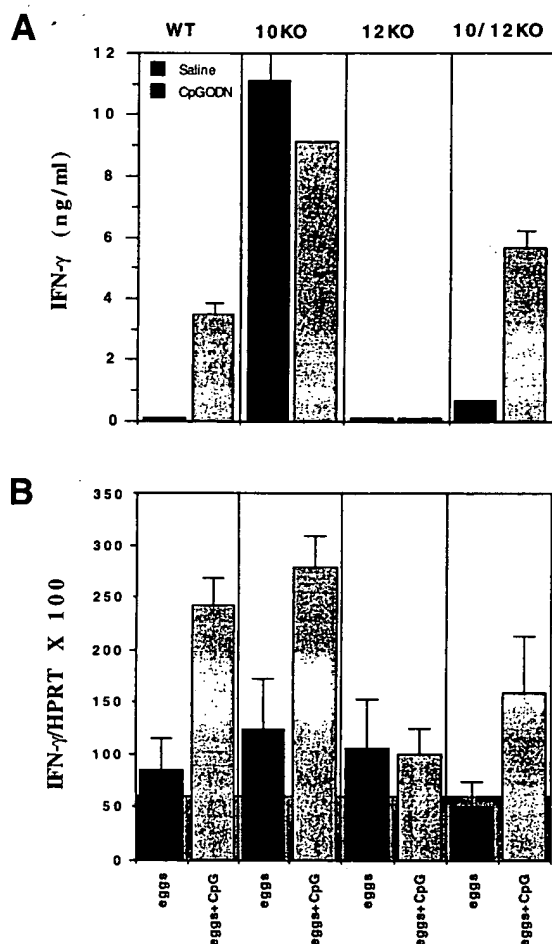


FIGURE 11. CpG ODN induced an IL-12-independent increase in IFN- γ expression in IL-10/IL-12 KO mice. **A**, WT, IL-10-, IL-12-, and IL-10/IL-12-deficient mice were sensitized and challenged as described in Fig. 10. Next, 72-h culture supernatants from LALN cells restimulated in vitro with SEA (20 μ g/ml) were analyzed by ELISA for IFN- γ . Bars represent means of supernatants analyzed in duplicate. **B**, IFN- γ mRNA was measured in the lung by RT-PCR. Bars represent means of six mice per group. The horizontal shaded bar represents the background IFN- γ /HPRT value of uninjected WT mice.

B7.1 and CD40 costimulatory molecule expression is increased in CpG-ODN-activated macrophages in the absence of IL-12 and/or IL-10

In an attempt to explain the unexpected increase in IFN- γ production in CpG-ODN-treated IL-10/IL-12-deficient mice, we examined the expression of several costimulatory and cell-surface markers after in vivo CpG-ODN treatment. For these experiments, WT, IL-10 KO, IL-12 KO, and IL-10/IL-12 KO mice were injected with thioglycollate and on day 4 postinjection were treated with saline or 30 μ g CpG-ODN i.p. All mice were sacrificed 24 h later, and B7.1, B7.2, MHC class II, and CD40 expression was examined on peritoneal-derived macrophages by FACS. As shown in Fig. 12, the macrophage population was selected by forward/side scatter (*left panels*, gate R2). Cells in the selected gate were ~93–97% F4/80 positive. In WT and IL-12 KO mice, the frequency of F4/80-positive macrophages was not affected by CpG-ODN treatment, whereas CpG-treated IL-10 and IL-10/IL-12 KO mice consistently had fewer F4/80-positive macrophages (~30% fewer) and increased numbers of lymphocytes and granulocytes when compared with their saline-treated counterparts. Many macrophages from all four groups were B7.1 positive and showed increased expression after CpG-ODN treatment, which was not significantly influenced by the absence of IL-12 and/or IL-10. In contrast, B7.2 expression was only partially up-regulated and to the greatest extent in IL-12-deficient mice. MHC class II expression was up-regulated in WT and IL-12 KO mice, while IL-10 (data not shown) and IL-10/IL-12 KO mice showed a less dramatic increase after CpG treatment. There was only a slight shift in CD40 expression in both WT and IL-12 KO mice by CpG-ODN. Strikingly, however, macrophages from the saline-treated IL-10 (not shown) and IL-10/IL-12 mice already displayed similar levels as the CpG-activated WT or IL-12 KO-derived cells and showed much higher levels after CpG-ODN treatment. These data strongly suggest that IL-10 is a potent inhibitor of CD40 expression. It should be noted that results from IL-10-deficient animals were omitted from the figure because the data were virtually identical with the double IL-10/12-deficient mice.

Discussion

There is growing interest in the use of unmethylated CpG oligonucleotides as vaccine adjuvants to promote protective Th1-type responses (13, 14, 20, 48). Indeed, studies in several infectious

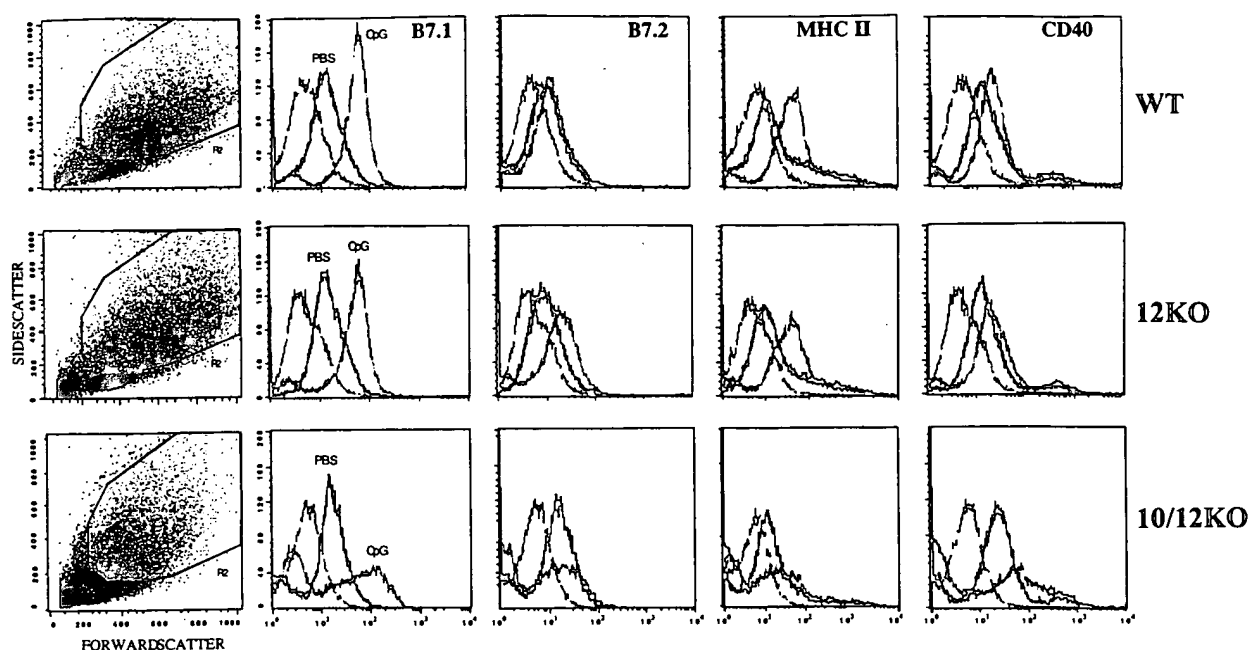


FIGURE 12. B7.1 and CD40 expression is increased on CpG ODN-activated macrophages in the presence or absence of IL-12 and/or IL-10. Thioglycollate-injected WT, IL-12-, and IL-10/IL-12-deficient mice were treated i.p. with CpG ODN (30 μ g). Cells were harvested 24 h later and analyzed by FACS for B7.1, B7.2, CD40, and MHC class II surface expression. The left panels show the forward/side scatter for the respective CpG-treated groups. The R2 gate used in the analysis contained the majority of F4/80-positive cells. The shaded histogram represents the unstained cells, and the black and gray outlines, respectively, show specific Ab staining in the PBS- and CpG-treated mice. This experiment was repeated multiple times with similar results.

disease models clearly demonstrate the marked ability of coadministered CpG ODN to enhance vaccine-induced cell-mediated immunity (18, 19, 49, 50). CpG DNA also down-regulated Th2 responses and bronchial hyperactivity in a murine model of asthma (34). In more recent work, it was concluded that CpG oligonucleotides likely exert their Th1-enhancing effects via IL-12 (49) and IFN- γ -dependent mechanisms (50). However, much less is known regarding the role of IL-12 or IFN- γ in the regulation of Th2 responses by CpG ODN. Our data demonstrate that while IL-12 clearly participates in CpG-induced immune deviation, significant reductions in Th2 cytokine expression and granulomatous inflammation occur in the absence of endogenous IL-12. The data suggest that CpG-induced IL-10 (25, 37) as well as B7.1 (51) and CD40 (52) expression might provide an additional mechanistic explanation for the suppression of Th2 responses in egg/CpG-sensitized IL-12-deficient mice. Thus, in contrast to its Th1/IFN- γ -inducing activity where IL-12 is essential (49, 50), the Th2-suppressing ability of CpG ODN appears to extend beyond a simple model involving the induction of only IL-12.

Our data show that sensitizing mice with relatively low doses of CpG ODN in combination with schistosome eggs protected animals from subsequent egg-induced pulmonary pathology (Fig. 1). The reduction in granuloma size was accompanied by an almost complete ablation of tissue eosinophilia and a shift in the normally pathogenic Th2-type response to a Th1-dominated reaction (Fig. 2A). Consistent with this pattern of immune deviation, serum IgG1/IgE Ab levels decreased and IgG2b titers were increased in the egg/CpG-sensitized mice (Fig. 2B). Thus, these data extend previous observations with rIL-12 (15, 27) and show that CpG oligonucleotides can duplicate many of the protective functions previously assigned to this central immunoregulatory cytokine. Indeed, to our knowledge this is the first demonstration of the prophylactic inhibition of Th2-dependent egg-induced pathology by CpG ODN. Moreover, our recent unpublished experiments showed

that CpG ODN/Ag sensitization was as effective as rIL-12 (28, 53) at reducing hepatic pathology in *S. mansoni*-infected mice and, similarly, enhanced protection elicited by the irradiated cercariae vaccine (data not shown).

Nevertheless, while CpG ODN produced similar results as rIL-12 (27), the mechanism of action of the immunostimulatory DNA is likely different from IL-12, by virtue of the different cells responding to each adjuvant. Indeed, macrophages and dendritic cells are major targets of CpG DNA (3, 8), while IL-12 primarily but not exclusively stimulates NK cells and T cells (32). IFN- γ - and IL-12-deficient mice were used to determine whether the Th2-reducing activity of CpG DNA was dependent on the induced expression of one or both of these cytokines. As seen previously with rIL-12, the granuloma/eosinophil-reducing effects of CpG DNA were highly dependent on IFN- γ signaling (33, 41), because no reduction in granuloma size or tissue eosinophilia was seen in the sensitized IFN- γ -deficient mice (Fig. 4A). In fact, consistent with previous findings with rIL-12, CpG ODN appeared to worsen pathology in the absence of IFN- γ (41). Thus, these findings are similar to recent results by Sur et al., where administration of CpG ODN in IFN- γ ^{-/-} mice failed to inhibit pulmonary eosinophil recruitment in experimental asthma (35). However, much more surprisingly, granuloma size was markedly reduced in the CpG/egg-sensitized IL-12-deficient mice, although it was never as complete as the reduction observed in WT mice. These findings suggested that CpG-induced IL-12 expression was not essential for Th2 response inhibition. This was unexpected because it was predicted that the induction of IL-12 by CpG DNA would be an important early requirement in the cytokine cascade leading to the deviated Th1 immune response and acquired resistance to egg-induced pathology.

However, it is important to point out that while granuloma size was reduced in the egg/CpG-sensitized IL-12-deficient mice (Fig. 4A), there was little or no reduction in granuloma eosinophils when

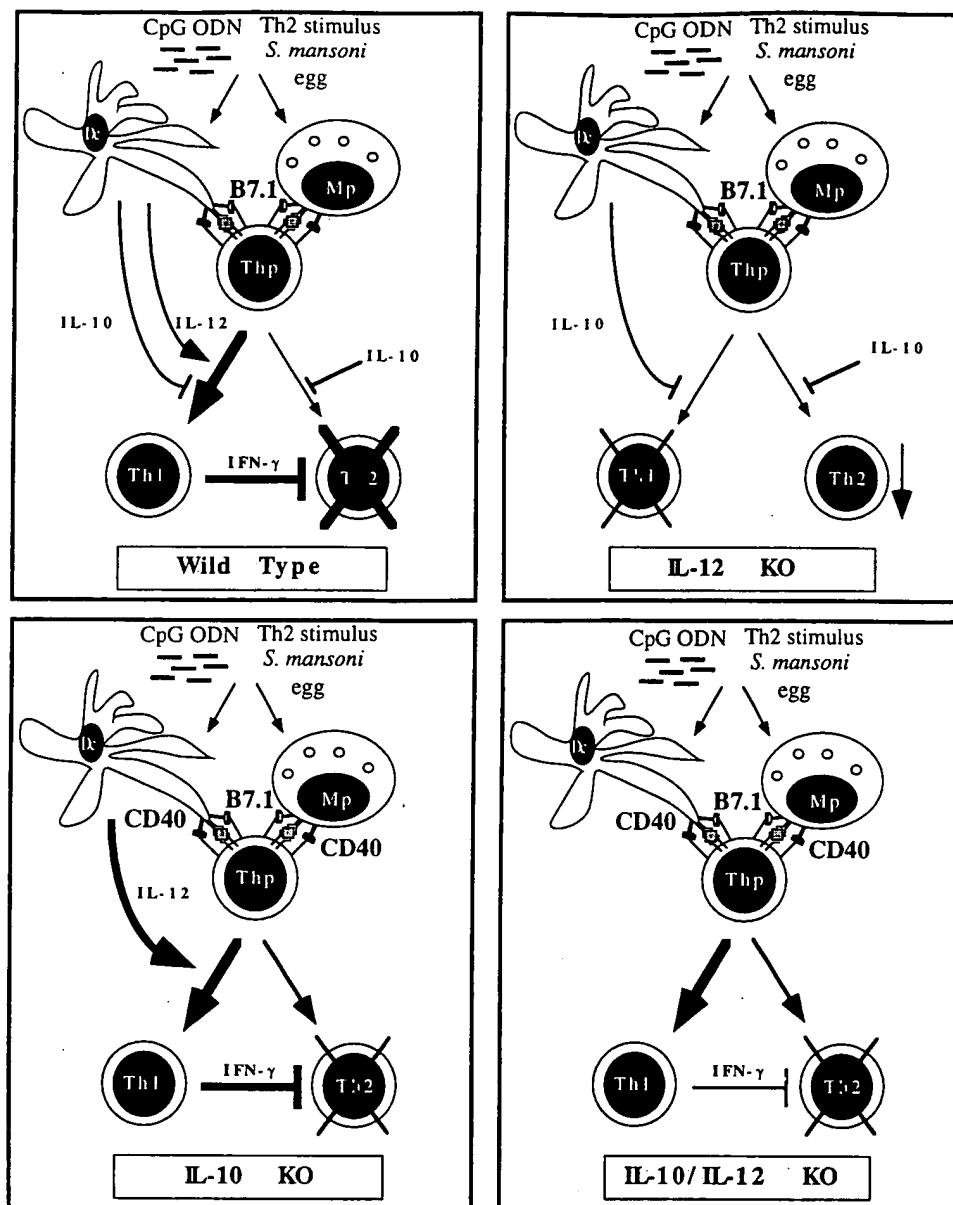


FIGURE 13. Regulation of Th2 responses by CpG ODN. *Top left panel*, In WT mice, CpG ODN induces IL-12 and IL-10. IL-12 drives an egg-specific Th1-type cytokine response, while IL-10 dampens the overall response, possibly by down-regulating costimulatory molecule expression (i.e., CD40). Again, the preferential up-regulation of B7.1 by CpG ODN may also aid in Th2 response inhibition. *Top right panel*, In the absence of IL-12, CpG ODN markedly increases IL-10 production by macrophages/dendritic cells, which dampens the evolving egg-specific Th2 response. Without IL-12, there is little or no induction of a Th1-type response. The preferential up-regulation of B7.1 vs B7.2 may also help antagonize Th2 cell development. *Lower left panel*, In the absence of IL-10, CD40 expression is markedly increased on CpG-stimulated macrophages, as is the production of IL-12. Together, these changes promote the development of a robust Th1-type response, which cross-regulates the Th2 response. *Lower right panel*, In the absence of IL-12 and IL-10, an IL-12-independent increase in IFN-γ-producing cells is observed in the egg/CpG-sensitized mice, which may be explained by the dual increase in B7.1 and CD40 expression in these animals. Here, the increased IFN-γ response contributes to the significant reduction in IL-4/IL-5-producing cells.

compared with the saline-treated controls (Fig. 4B). This is in marked contrast to WT mice where egg/CpG sensitization reduced granuloma size as well as the number of lesion-associated eosinophils. IL-5 is essential for the tissue eosinophilia in this model (43, 54), and IFN-γ is an equally potent inhibitor of the response (41). These observations suggested that there was no general Th2 to Th1 switch occurring in the CpG-sensitized IL-12-deficient animals. As expected, IL-12- and CpG-treated WT mice showed a marked increase in IFN-γ and decreased IL-5 production (Fig. 5).

These findings were completely consistent with the observed effects on pathology in these mice (Fig. 4). However, more surprising was the observation that Th2 cytokine expression was reduced in the CpG-treated IL-12-deficient mice. This was somewhat unexpected because there was no corresponding increase in IFN-γ expression. The latter observation is consistent with recent findings by Halpern et al., who showed that stimulation of IFN-γ production by bacterial DNA is highly dependent on IL-12 (55). Nevertheless, these findings fail to explain the markedly decreased Th2

response in the CpG-treated IL-12-deficient mice, because cross-regulation by IFN- γ -producing Th1 cells does not appear to be a probable mechanism (56).

NK cells are also activated by CpG DNA (5), and transient production of IFN- γ by these cells during the period egg/CpG sensitization could also provide a possible mechanism for the reduced Th2 response observed in the IL-12-deficient mice. In many infectious disease models, the rapid production of IFN- γ by NK cells can have a significant impact on the evolution of CD4⁺ Th1/Th2 cell responses (57). We investigated this possibility by depleting NK cells during periods of sensitization in both WT and IL-12 KO mice. However, the results from these studies indicated no significant contribution by NK cells. Granuloma size and tissue eosinophilia were completely unaffected by the absence of NK cells (Fig. 6), and there was also no effect on IFN- γ or IL-5 production in either group (data not shown). While B cells are also targets of CpG DNA (10), they too appeared to play no necessary role in Th1/Th2 response polarization (Fig. 3). The latter conclusion is consistent with previous findings that suggested B cells are not critical for CD4⁺ T cell priming (42).

While NK cells and B cells appeared to play little or no role, our data do suggest at least a partial role for IL-10 in the CpG-induced down-regulation of Th2 responses in the IL-12-deficient animals. Indeed, IL-10 was induced in CpG ODN-activated macrophages derived from both WT and IL-12-deficient animals (Fig. 8). In vivo experiments also showed a marked up-regulation of IL-10 mRNA coincident with IFN- γ in the granulomatous tissues of CpG ODN-treated mice (Fig. 7A). Recently, we showed that IL-10, in addition to down-regulating Th1 responses, also suppresses *S. mansoni*-induced Th2-type responses (25, 37). Therefore, the maintenance of the CpG-induced IL-10 response in the absence of IL-12 may in part explain the surprising IL-12 KO result. Moreover, the much less pronounced IL-10 response observed in CpG-stimulated IFN- γ KO macrophages is consistent with the near complete failure to suppress Th2 cytokine expression in those animals (Fig. 8). These findings suggest that stimulation of IL-12/IFN- γ , as well as IL-10, provide at least two related mechanisms to regulate Th2 response development by CpG ODN. WT mice would benefit from both regulatory pathways (Fig. 8), while IL-12-deficient mice, which showed almost no Th1-type response (Fig. 5), could regulate the magnitude of their egg-induced Th2-type response by up-regulating macrophage- and/or dendritic cell-derived IL-10 in response to CpG ODN.

Consistent with a combined inhibitory role for IL-12 and IL-10, the egg/saline-sensitized double IL-10/IL-12 KO mice showed a striking increased frequency of Ag-specific IL-4- and IL-5-producing cells (Fig. 10), when compared with the individual cytokine KO or WT mice. Interestingly, however, all four groups displayed a significant reduction in IL-4 and IL-5 producers when sensitized with eggs in the presence of CpG ODN. This was accompanied by a marked reduction in granuloma size, although the CpG-sensitized IL-10/IL-12-deficient mice failed to achieve a significant reduction (Fig. 9). However, stimulation of IL-10 production by CpG ODN in IL-12 KO animals appears to only partially explain the suppressed Th2 response, because the double IL-10/IL-12 KO mice also showed a marked reduction in IL-4- and IL-5-producing cells (Fig. 10). It is important to note that the double KO's never attained the nearly complete Th2 ablation that was observed in the CpG-sensitized WT mice. In fact, the frequency of IL-5-producing cells equaled that seen in the egg/saline-sensitized WT mice, which likely explains the maintenance of a significant Th2-mediated granulomatous response in the egg/CpG-sensitized double KO mice.

Most surprising from these experiments was the finding that the frequency IFN- γ -producing cells were increased in the CpG-treated IL-10/IL-12-deficient mice (Fig. 10). IFN- γ levels were also up-regulated in the supernatants of cultured lymph node cells (Fig. 11A) and IFN- γ mRNA expression was similarly increased in the granulomatous tissues (Fig. 11B). Again, this was in marked contrast to the single IL-12-deficient mice, suggesting that the additional deficiency in IL-10 revealed a potentially novel IL-12-independent pathway to induce IFN- γ production. The Ag-specific IFN- γ response in the CpG-sensitized double KO mice likely explains the reduced number of IL-4/IL-5-producing cells detected in those animals.

The studies with the double cytokine-deficient mice indicated that factors other than IL-10 were also likely involved in the suppression of Th2 responses in the egg/CpG-sensitized IL-12-deficient mice. Changes in macrophage/dendritic cell costimulatory molecule expression could also significantly impact on Th1/Th2 cell development (51, 58–60). Indeed, recent studies with dendritic cells showed a marked up-regulation of MHC class II, CD40, and B7 costimulatory molecule expression after in vitro stimulation with CpG ODN (8). We found a similar expression pattern in thioglycollate-elicited macrophages activated in vivo with CpG ODN (Fig. 12). Interestingly, B7.1 was up-regulated to a much greater extent than B7.2, and IL-12 and IL-10 appeared to play no role in this response, because similar changes were observed in the WT, IL-12 KO, IL-10 KO, and IL-10/IL-12-deficient mice. A slight increase in CD40 expression was also observed with WT and IL-12-deficient cells, although a more dramatic shift was detected in CpG-treated IL-10 and IL-10/IL-12-deficient animals. This suggested that CpG-induced IL-10 down-regulates CD40 expression. In contrast, IL-12 had no influence on CD40 levels because WT and IL-12-deficient mice displayed the same expression pattern. Selective expression of B7.1 vs B7.2 has been shown in many models to preferentially influence Th1- and Th2-type responses, respectively (51). In addition, CD40 is an important cofactor in IL-12-induced Th1 responses (61). These data suggest that a marked up-regulation in B7.1 and CD40 expression in egg/CpG-sensitized double IL-10/12-deficient mice might contribute to the IL-12-independent increase in IFN- γ expression in these animals (see Fig. 13). Nevertheless, a direct IL-12-independent role for CD40 in Th1 response development has not been previously described. However, consistent with this hypothesis, the much less dramatic increase in CD40 expression observed in IL-12-deficient mice might explain the inability to generate a Th1-type response in those animals. The preferential up-regulation of B7.1 vs B7.2 in CpG-treated animals (Fig. 12) could also negatively influence Th2 cell development and provide at least one additional explanation for the down-regulated Th2 response in both WT and IL-12 KO mice. Nevertheless, specific blocking studies in the various cytokine-deficient animals will be needed to confirm these hypotheses.

In summary, our data demonstrate that multiple factors are involved in the prophylactic inhibition of Th2 responses by CpG ODN, including IFN- γ , IL-12, IL-10, B7.1, and CD40. Th2 cytokines influence susceptibility to many infectious diseases and are also the major mediators of allergy and asthma. Thus, the data presented here demonstrate the enormous potential of CpG oligonucleotides in immune deviation strategies aimed at reducing Th2-dependent pathology. Moreover, they illustrate that a simple paradigm involving the initial production of IL-12 is insufficient to fully explain the many functions of CpG ODN on the immune response. Indeed, these data show that CpG DNA exhibits many important activities that are completely IL-12 independent.

Acknowledgments

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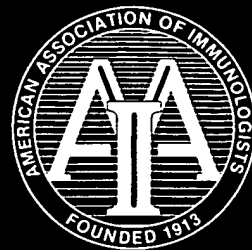
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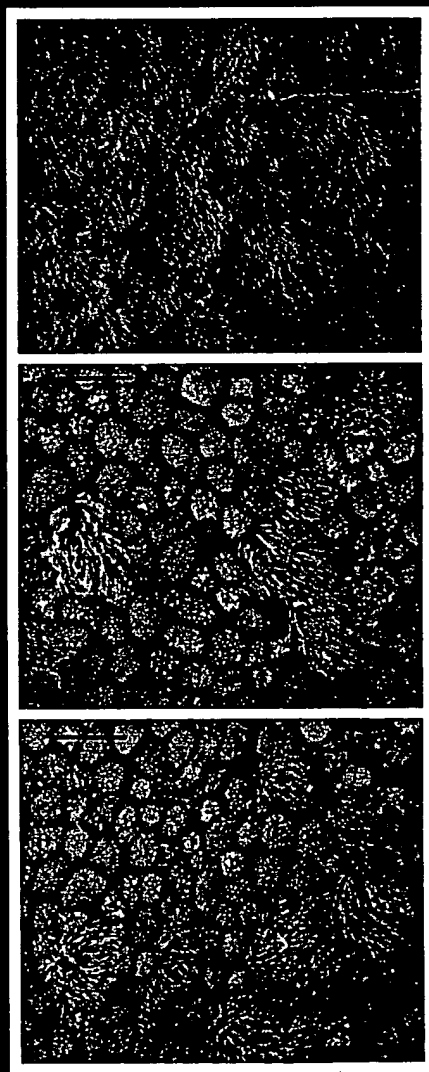
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